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Contact: Prof. Bassam Z. Shakhashiri

UW-Madison Department of Chemistry

bassam@chem.wisc.edu

www.scifun.org

H-NS–mediated mechanisms of transcription elongation silencing and counter-silencing in K-12 and uropathogenic *Escherichia coli*

By

Christine Michelle Hustmyer

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The dissertation is approved by the following members of the Final Oral Committee:

Robert Landick, Professor, Biochemistry and Bacteriology
Katrina Forest, Professor, Bacteriology
Melissa Harrison, Professor, Biomolecular Chemistry
Christina Hull, Professor, Biomolecular Chemistry and Medical Microbiology & Immun.
M. Thomas Record, Jr., Professor, Biochemistry and Chemistry

Chapter 5: The Bacterial Transcription 500 Mile Race: How bacterial chromatin proteins control the race of bacterial transcription

C.M.H. wrote this chapter with the aim of describing her research to a non-science audience as part of the Wisconsin Initiative for Science Literacy (WISL). C.M.H. is grateful for the WISL director Professor Shakhashiri, Cayce Osborne for coordinating the WISL program and toWISL editor Elizabth Reynolds for helpful comments on this chapter. C.M.H. credits the idea of comparing her research to the Indy 500 to her committee member Professor Melissa Harrison. C.M.H. is also thankful to Mr. Nathan Pfeffer, who calculated the estimate for the number of bacteria that could fit inside the Indianapolis Motor Speedway. And of course, C.M.H. is thankful for her Dad Frank G. Hustmyer, for sparking the love of the Indy 500 when she was 2 years old.

5.1 Abstract of 'H-NS—mediated mechanisms of transcription elongation silencing and counter-silencing in K-12 and uropathogenic *Escherichia coli*' for a general, non-scientific audience

Bacteria are all around us: in the foods we eat, the objects we touch, and in the bodies we live in. They are small, and because they are small, they often get overlooked as being simple. In reality, bacteria use a myriad of DNA sequences, called genes, which encode factors called proteins, to control their behaviors. When certain genes are turned on to encode proteins, these proteins help the bacteria to answer questions like: Should I grow here? Should I die now? Should I release this toxin to survive? Should I allow this foreign DNA to be incorporated with my DNA? Should I impede the expression of another factor so I can stay plentiful in number? Sometimes genes are turned off, which can further influence bacterial behavior. It has been my job in my Ph.D. to tease apart the elegant means by which bacteria have evolved to turn genes on or off to influence their behavior. I love bacteria, which is why I have spent the past six years working with them. But I also have another love: attending the Indianapolis 500-mile auto race with my Dad. Watching auto-racing has been one of the saviors of my Ph.D. (as has the constant advice of my dear Dad). Thus, it only seems fitting that in summarizing the important findings of my Ph.D. to a general audience, I should attempt to describe my two niche hobbies, bacteria and auto-racing, in the same chapter. In this chapter, I describe the process of my Ph.D. using extended auto-racing analogies in three parts, including *i*) why bacteria and bacterial transcription elongation matter, *ii)* what I have discovered about how transcription contributes to bacterial behavior in my Ph.D., and *iii)* what I have learned from studying bacteria during my Ph.D. about my own life.

5.2 Why I wrote this chapter (or "What do race cars have to do with bacteria anyways?")

You may have not heard of the intricacies of bacterial transcription. I like to think of transcription as an orchestra performing a complex-to-listen-to symphony. In bacterial transcription, there are hundreds of proteins working together (or sometimes against each other) to complete an end goal of gene expression. Transcription, which controls gene expression, is the process by which bacteria convert their DNA sequences (genes) into messenger RNA (mRNA), which will then be converted to proteins, which then control bacterial behaviors. The goal of my research has been to understand how gene expression impacts bacterial behaviors, so we better understand how bacteria contribute to human health and other processes in the world.

These past six years have been filled with science. I take great pride that I can talk your ear off about bacteria, which may be unique at certain dinner parties. But perhaps even more unique is my ability to wax poetic about my love of the Indianapolis 500. If you talk to me for more than five minutes, you might notice my race car necklace. You will learn what the "Indy 500" is not a NASCAR race, but instead a thrilling over-100-year-old tradition that families from across the world share. And if you know me long enough, you will learn that the Indy 500 race is a tradition I have shared with my own Dad, my best friend, since I was 2 years old.

You also may have not heard of the intricacies of the Indianapolis 500. Well, today is your lucky day, as this is a two-for-one chapter on both bacterial transcription and the Indy 500! The Indy 500 is a glorious race where 33 drivers compete for immortality. The racecourse, a 2.5-mile oval with 9.2° banking, in which cars turn left four times to complete one lap, for a total of 500 miles, is the pride of Indianapolis. This race is a quest to test the limits of humankind and machines. For over 100 years, this race has been the pinnacle of innovation. In 1911, the first rear view mirror for a car was developed here and in 2023, blistering speeds of over 240 mph were reached. Strategy, intelligence, chutzpah, and risk-taking are all key components to a

successful Indianapolis 500-mile race (traits of which I have found are also essential to my Ph.D.).

Every year since 1998 I have attended this glorious race with my Dad. My Dad has been my #1 fan, sponsor, and crew-chief all my life. My Dad raised me as a single parent, often having to sacrifice the progression of his own scientific career to do so. Because my Dad is a scientist, I wanted to pursue science so I could be like him. My Dad also passed on a shared passion for the Indy 500. My first exposure to the Indy 500 was when I was 2 years old. I was bribed to attend with ice-cream sandwiches (I had 4, reportedly) and the promise of sighting a purple race car. And since age 2, I was hooked. Race day is the fresh smell of burning ethanol (the fuel the cars have used for the past decade, because the Indy 500 also looks to innovate scientifically!), the roar of jets during the pre-race flyover, the shouts of "WHAT DID YOU SAY" over the hum of 700 horsepower engines that appear to turn left perpetually, and the pain of whipping your neck back and forth to catch a glimpse of the rockets-on-wheels that pass by you going into turn one; just recalling these feelings bring chills to my spine. The Indy 500 is akin to a religious holiday to us. And looking forward to the Indy 500 with my Dad has been a happy spark, especially in my Ph.D.

As I finished my Ph.D., I realized that my journey in many ways parallels the Indianapolis 500-mile auto race. I was often a strategist, sometimes an overly emotional fan, sometimes a pit-crew member who could not properly inject fuel into a racecar, and occasionally an engineer who poured over data plans late into the evening. Sometimes, I started the race too fast and burnt out my tires. I was unfortunately never a wealthy team owner. But I was always the driver. And while I may not ever feel as if I won the Indianapolis 500, or "won" my Ph.D., I am happy to know that I have finished. The goal of this chapter is to explain my scientific work

and the journey of my Ph.D. to a non-scientific audience. To do so, I will compare my research to my life's biggest passion: the Indianapolis 500-mile race.

So, welcome to my extended analogy of the Indianapolis 500, the process by which cars finish 500-miles, and bacterial transcription, the process by which bacteria finish a race to complete a certain behavior. I will present to you an introduction of the participants in this race (factors of interest) before summarizing the conclusions made from each individual race (experiment) and conclude with a post-race perspective on what I learned in my Ph.D.

5.3 Why should you care about something you cannot see? The case for studying fascinating bacteria

5.3.1 Bacteria are everywhere

Many of us have heard of bacteria. We know that bacteria can cause diseases (such as Urinary Tract Infections or Food Poisoning). We know from yogurt commercials online that certain bacteria can promote a "healthy gut". But we cannot see bacteria with our naked eye. We cannot, for example, see how many bacteria coat the computer screen upon which you are currently reading this sentence. And therefore, the importance of studying bacteria may be overlooked.

To lend some perspective on how small bacteria are, I will first compare bacteria size to humans. The area of one of the bacteria I work with in the lab, *E. coli,* is 2.0 × 0.5 μm in length and width, respectively. The average size of a human in length is 2 meters, so about one million times larger in length. To further compare the sizes, please allow me to incorporate the Indianapolis Motor Speedway, which is considered one of the largest oval-tracks in the United States. On race day, 300,000 people flock to sit around the 253-acre infield of its colossal track. By contrast, 1×10^{18} bacteria can lay flat inside the Indianapolis Motor Speedway infield (**Figure 5.1**). We are dealing with some very small organisms here!

A common misconception about bacteria is that because they are small, they are simple. But there is much to uncover about bacteria and their function. For example, there are many different species of bacteria that live in different environments. These bacteria can provide good functions, such as biofuel production, or enable food production under extreme environmental conditions.[1] By studying different bacterial organisms from unique niches, we can uncover how bacteria contribute to the world around us.

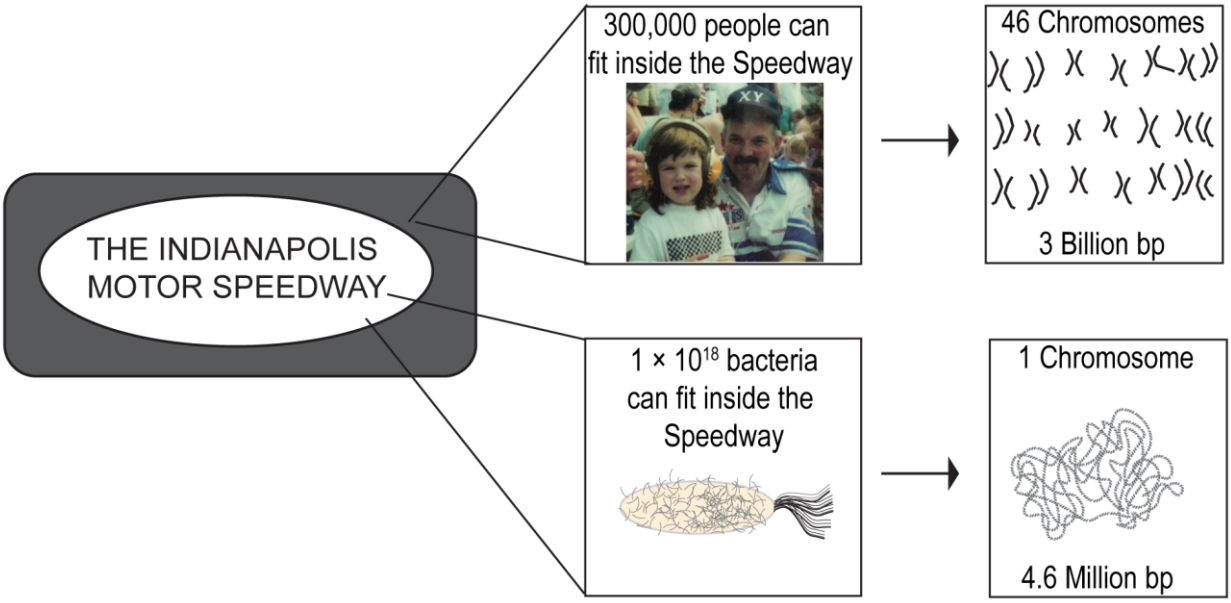

Figure 5.1. Bacteria vs. humans: A scale cartoon using the Indianapolis Motor Speedway as reference.

Top: 300,000 humans can fit inside the Indianapolis Speedway, represented by my Dad and I at the race track in 1998 (my first race!). My Dad has 46 chromosomes and a genome of ~3 billion base pairs (bp). Bottom: The bacterium I study called *E. coli* is represented by a tan oval, hairy-like particles are the flagella. If E. coli lay flat, 1×10^{18} can fit inside the oval. An *E. coli* bacterium has one chromosome of 4.6 million base pairs.

5.3.2 Bacterial transcription controls how bacteria behave

What makes one bacterial species different from another? The answer is its genome, or the order of genetic sequences of ATCG (nucleotides) that make up and store genetic information in a molecule of DNA. The bacteria I study, *E. coli*, has just one genome, or chromosome, whereas humans have 23 pairs of chromosomes in total. Human chromosomes are much larger (~3 billion base pairs (bps) in total compared to bacterial chromosomes (~4.6 million bps) (**Figure 5.1**). Once again, you may think that this makes bacteria too simple to study, but despite its smaller size, the bacterial chromosome is still quite active and complex. For example, we know that sequences of DNA can be grouped together to encode for a certain behavior. One bacterial species may contain a long sequence of ACGTs that is not present in another bacteria, and thus makes the bacteria distinct and able to perform different behaviors. We call these sequences that encode for different proteins that permit a bacterium to behave in a certain way "genes".

What happens to these genes that allow bacteria to behave in a certain way? Bacteria use a factor called RNA polymerase to decipher the sequence information from genes in a process known as transcription. RNAP reads a DNA gene into another messenger molecule, called mRNA, which can then be read-out by another factor called a ribosome (**Figure 5.2A**). This ribosome converts this mRNA sequence into a protein (for example, the RNAP and the ribosome are proteins) which can lead to different outcomes. Some proteins can interact with and bind to DNA sequences. Others can attack human cells when bacteria invade our bodies. Others can speed up reactions. Thus, proteins have many different functions, but help bacteria to accomplish certain goals and behave in certain ways.

In my thesis work, I mostly focus on one step of the complex bacterial behavior puzzle, transcription elongation. In the Indianapolis 500, a race car must qualify to be the fastest of 33

drivers to even start the race. So, I, too, focus on the start of this process because if the goal is to make a protein, one must first make mRNA. The goal of RNAP is to complete a round of transcription, successfully traversing the length of a DNA molecule, to create a full-length mRNA (**Figure 5.2A**).

What does a successful (but simplified) round of bacterial transcription look like? There are three steps: initiation, elongation, and termination (**Figure 5.2B**); to help distinguish these steps, I will compare each step of transcription to the steps my Dad and I take to drive to the Indianapolis Motor Speedway on race day. The first step, initiation, occurs when RNAP recognizes a certain sequence near a gene on the DNA called a promoter. This is the "start". For example, my Dad and I initiate, or start, the process of driving to the Indianapolis Motor Speedway when we prepare for the day. We ready ourselves with sunscreen, Diet Coke, a cooler of frozen pizza, our earplugs, and merchandise of a favorite driver. We make sure enough gas is in the car. And then we get in the car and start the 25-minute drive to the Indianapolis Motor Speedway. Starting the engine starts our process, just as RNAP binding to a promoter initiates transcription (Figure 5.2B). The start is never as simple as getting in the car and heading off—we had to prepare and make sure we had the necessary materials. In summary, bacteria must do the same when they start transcribing DNA. They need certain materials to begin transcription (such as RNA polymerase, a key initiation factor called Sigma, sometimes other factors called initiator proteins to guide RNAP to the DNA, and nucleotides so they can convert the DNA into mRNA).

Next, RNAP elongates, transcribing the DNA into mRNA (**Figure 5.2B**). This is similar to my Dad and I driving to the Indianapolis Motor Speedway; we've already started and now we are completing the actual task. There is usually a lot of traffic on our way to the speedway, a lot of starts and stops. RNAP encounters a similar phenomenon when elongating, it often pauses on

certain DNA sequences (orders of ACTGs). These pauses can be key regulatory features of transcription.[2] For example, if RNAP pauses for too long, RNAP may not finish transcription. This is like a car in traffic: if you stall in the middle of the highway because you are out of gas, you will not reach your destination. Sometimes another car cuts you off and you crash, which prevents you reaching your destination. The same can happen with RNAP: other factors can slow RNAP down by binding in front of RNAP, preventing RNAP from completing its job of elongation. Understanding how RNAP elongates efficiently is a key part of my thesis work.

Finally, when RNAP reaches the end of the transcription, the mRNA is released and ready to be translated into protein (**Figure 5.2B**). Transcription has been terminated: the goal is complete. This is similar to my Dad and I arriving at our secret parking spot with an easy-out at the Indianapolis Motor Speedway. We reached our destination-it was a smooth ride. But what happens when the ride is less smooth (*e.g.* when there is a lot of traffic)?

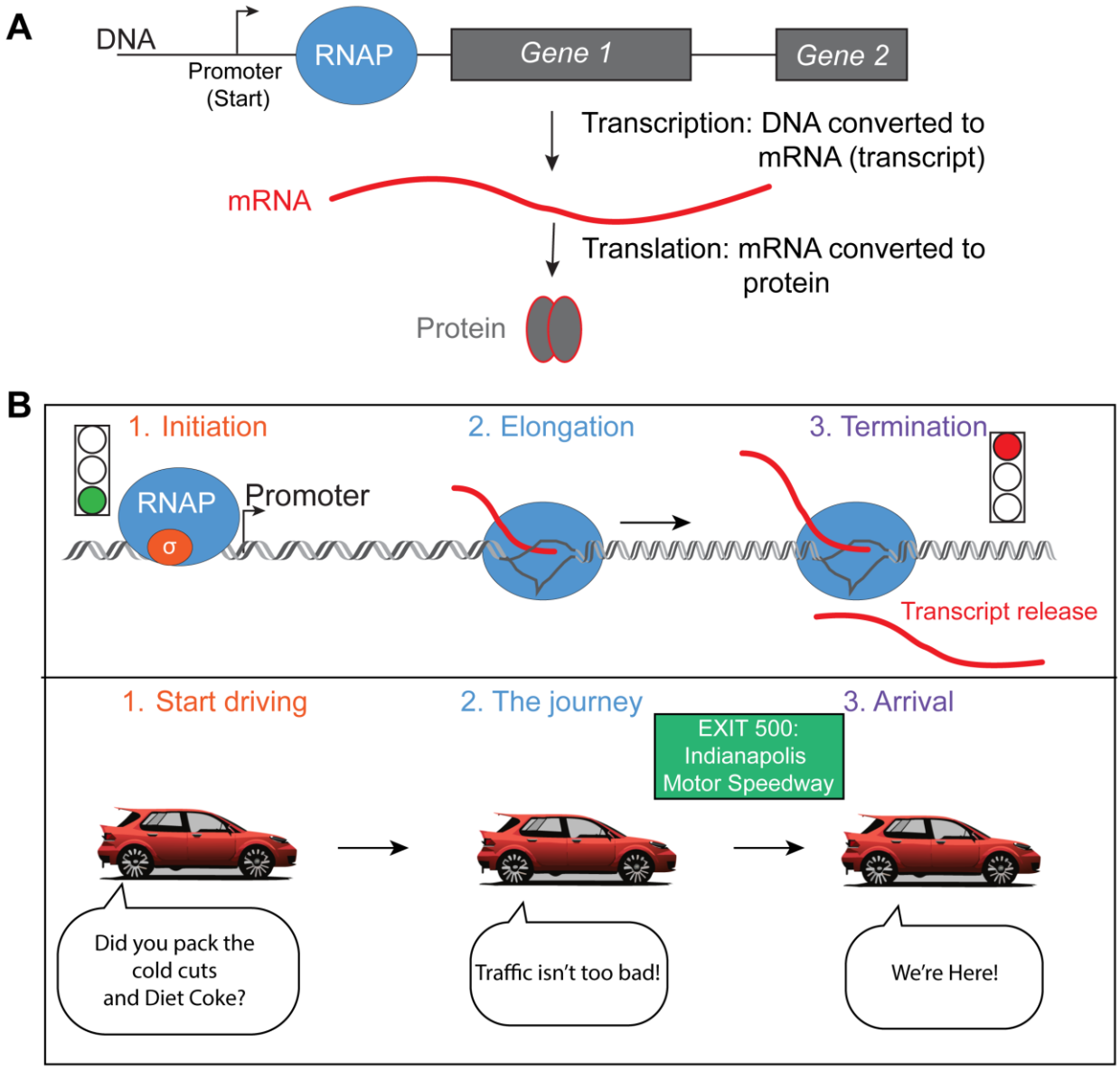

Figure 5.2. The role of transcription in protein synthesis and the steps of transcription.

A: DNA contains sequence elements like promoters (arrow), which tell RNAP (blue circle) where to start transcription, and genes (gray boxes), which encode for proteins. RNAP carries out the process of transcription by converting DNA into mRNA (red). mRNA can then be converted to protein. B: The steps of transcription. Transcription begins when RNAP and a Sigma factor (orange) bind to a promoter DNA sequence. Similarly, we begin driving to the Indy 500 once we have all of our items for the race track. RNAP elongates (continues driving to destination) and transcribes DNA (gray) into mRNA transcript (red). When RNAP reaches the end of a gene, the transcript is released, transcription is complete

(similarly to when we reach our destination). Cars were generated using the AI Image Generator from Adobe Illustrator.

5.3.3 Bacteria control transcription efficiency using proteins that can aid or hinder transcription

One of the main goals of my thesis work was to understand how bacteria affect transcription, specifically transcription elongation, to control their behaviors. How do bacteria deal with a less-than-smooth ride of transcription? In other words, 1) When is transcription unsuccessful or successful? Why? and 2) What proteins make transcription unsuccessful or successful? This allows me to ask deeper questions like, when does this bacterium that I know causes disease in humans turn on a certain group of DNA sequence genes, called an operon, to cause a harmful bacterial behavior to be turned on? Do bacteria naturally develop ways to keep a bad bacterial behavior turned off?

To understand these questions, I had to study proteins that act on RNAP elongation. There are proteins that specifically bind to RNAP to enhance RNAP elongation. There are also many proteins found in bacteria that can enhance or hinder the success of RNAP completing elongation (i.e. the ability of RNAP to reach the destination). I focused on a group of proteins called "bacterial chromatin proteins" or BCPs.[3] BCPs are interesting because they can bind to the same DNA RNAP is transcribing. I like to think of BCPs as construction workers on the highway, because on these DNAs they build different architectures, alternate routes, and unplanned exits on the bacterial genome. The effects of the structures these proteins build can either enhance or hinder RNAP progression of transcription elongation.

Understanding BCP and other protein regulation of RNAP transcription is more like a realistic long trek to the Indianapolis 500 (not the simplified version I described in the earlier section). BCPs can regulate the start of transcription (initiation), but I have focused most of my study on BCP regulation of elongation (driving to the track). On race day, 300,000 people flock to the Indianapolis Motor Speedway in the small town of Speedway, Indiana. So, one can

imagine, getting into the Indianapolis Motor Speedway is a pain. There are a lot of traffic jams on the highways, resulting in cars that are stalled, resulting in the inhibition of the speed at which my Dad and I can arrive at the race track. Similarly, in bacteria, there are proteins that bind DNA, blocking efficient RNAP transcription and slowing its progress (**Figure 5.3A**). I study how these proteins act to impede the progress of transcription.

On the other hand, there are also proteins that enhance transcription, enabling RNAP to complete the task of elongating (**Figure 5.3B**). Let's take the same traffic jam scenario of the trek to the Indianapolis 500. Some lucky few, including celebrities and well-to-do society members, are offered a police escort, allowing passengers to arrive at the Speedway with minimal traffic. The police escort is like proteins that can bind to RNAP to enhance elongation.

A big part of my thesis work has been to connect how proteins that hinder and enhance transcription work together or against each other in the bacterial cell. We can think of this interplay with the same highway analogy. Even when a car is transported by a police escort, the car still is interacting with the traffic that is backed up on the highway. In bacteria, almost every DNA sequence has some sort of protein associated with it that can either enhance or hinder transcription. It is my goal as a researcher to build this map and to understand where and when transcription is enhanced or hindered, which ultimately helps us understand how bacteria behave.

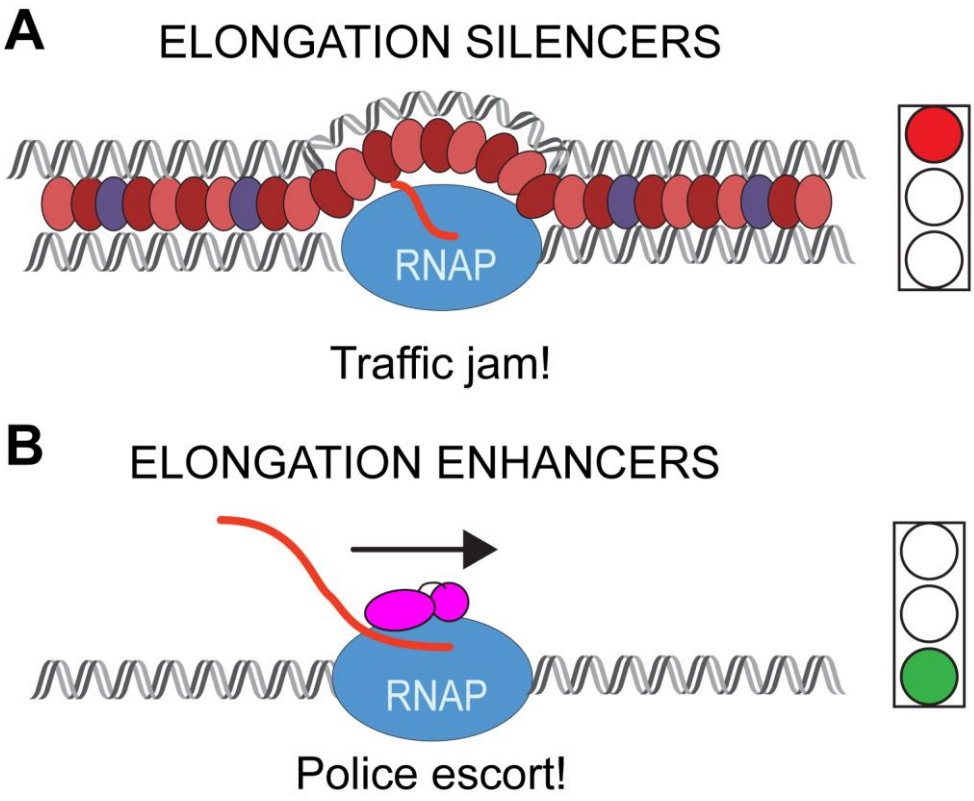

Figure 5.3. Examples of proteins that hinder or help RNAP elongation.
A: RNAP can encounter a traffic jam when encountering certain proteins (red and purple circles) that block forward progression. These proteins can also build complex structures on the DNA, like by bringing two DNA (gray helices) together. B: Elongation enhancers (pink protein) may bind to RNAP to help RNAP speed up transcription, thus acting like a police escort and enabling RNAP to reach the end of transcription faster.

5.3.4 H-NS slows transcription elongation in the lab, but what about in the cell?

The main protein I chose to study in my Ph.D. is a protein called H-NS. H-NS generally opposes the process of transcription (*i.e.* turns off gene expression), but the complete way it does so is less clear. We know from work done in the lab *in vitro* (containing isolated H-NS, DNA, and RNAP) that H-NS forms different types of structures on DNA. Linear (bound to one DNA) and bridged (bound to two DNAs) both seem to impede the start of transcription (**Figure 5.4**). Only bridged structures appear to impede elongation in these lab experiments.[4]

The way I think of this is that when H-NS binds to one piece of DNA (what we call linear), RNAP is blocked from accessing the DNA (transcription cannot start); but once RNAP is on the DNA, the linear H-NS filament is less adept at stopping its progress. This is like the city of Speedway closing certain roads after the race because too many inebriated pedestrians are exiting the racetrack. Pedestrians can get through, but cars cannot. Thus, you can think of H-NS binding in this linear fashion as slowing the process. Some RNAPs cannot start transcription, but if transcription has already begun, there is a way through.

Bridging is a bit more complicated. Now, normally, the colloquial phrase "building bridges" denotes a positive connotation; two points are connected, travel is easier. But in this case, H-NS builds a bridge that slows RNAP. Imagine a bridge on a highway filled with cars in both lanes that are stalled: no one can pass through. In your excitement for the Indy 500, you may honk your horn, but to no avail: you are permanently stalled. You may then get rear-ended by a car behind you who is grabbing a cold-cut from the cooler for pre-Indy 500 nourishment and not paying attention. Now, you are not making it to the race: your drive is terminated. Thankfully, my Dad and I have never encountered this analogy, but it helps to visualize just what H-NS appears to do in the lab. H-NS can act on elongating RNAP in different ways.

We also know that *in vitro* partners of H-NS called StpA, YdgT, and Hha appear to strengthen bridging to further impede RNAP elongation (**Figure 5.3**).[5, 6] You can think of this as adding more cars to the traffic jam: if the cars are already stalled, and then here comes three other cars trying to get into your lane, you will not make very good progress.

Why do we care about H-NS? Well, H-NS is found in all sorts of bacteria: from the bacteria that promote healthy gut health, to the bacteria that cause deadly diseases in humans. In H-NS, we have a direct protein that can stall transcription. As a scientist, I want to ask: how can I use this protein for our benefit? How can we manipulate H-NS to promote good behavior in bacteria and keep the bad behaviors turned off? To answer these questions, we need to know more about how H-NS works in cells. Most of our observations about H-NS were done in lab conditions, where we had purified DNA, RNAP, and H-NS; in cells there are 1000s of other components at play, including varying environments and conditions where the bacteria is growing (*i.e.* urinary tract of a human vs. the surface of a computer screen). I need to ask fundamental questions about how H-NS behaves in cells.

I will next describe the three major research questions of my Ph.D. Each of the Indy 500 races I have attended has been different: with a different winner, different crashes, different excitement. These three projects are like three individual races. The first race: What factors help RNAP to drive through H-NS? Said a different way, what allows a race car to withstand a crash and continue to finish the race? Second race: what does H-NS look like at the structural level; can we solve a picture of H-NS to better understand how it works? Said a different way, can I improve the clarity by which I take a picture of a race car traveling at 230+ mph? Third race: what factors enhance the ability of H-NS to silence transcription and are those factors necessary

for silencing transcription? In other words, if a race car encounters debris from a small crash, can the race car still finish the race?

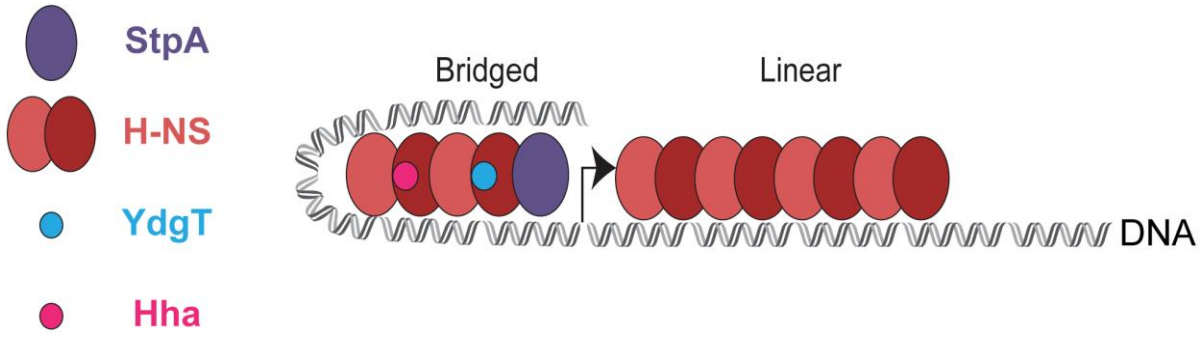

Figure 5.4. Cartoon model of H-NS filament.

H-NS (pink and red circles) can bind to DNA (gray) in a bridged (bound to two dsDNAs) or linear (binds to one dsDNA) structure. StpA (purple), YdgT (blue) and Hha (pink) can also bind with H-NS and appear to favor bridged structures.

5.4 What factors help RNAP to drive through H-NS?

5.4.1 Race 1: How does RNAP overcome H-NS silencing in bacteria that cause disease in humans?

As a researcher, one of the central questions I asked was what makes a pathogenic bacterium (a bacteria that causes disease) different from a bacterium that does not cause disease. I studied one species of bacteria in my research, a species called *Escherichia coli.* In my primary lab, I mostly worked with a strain (order of sequence) of bacteria called K-12 bacteria. This is a model organism, meaning many labs work with this bacterium. K-12 is also useful to work with in the lab because it has had its DNA sequence altered to remove or make inactive the DNA sequences that can harm humans. Thus, it is safe to grow and manipulate in the lab. Additionally, I wanted to work with *E. coli* that can cause diseases in humans to ask how transcription differs in the lab strain compared to the pathogenic strain. For this bacterium, I studied a strain called CFT073, which was isolated from a patient with a deadly ascending Urinary Tract Infection (meaning the bacteria spread from the bladder, to kidneys, to the blood)[7-9]. We wanted to ask *i)* how H-NS functions in this pathogenic bacterium strain and *ii)* what factors, if any, allowed RNAP to overcome H-NS based silencing to turn on certain genes that may harm humans.

So, I assembled my team and set out to complete the first race of my Ph.D. to answer these questions. This work was accomplished mainly in 2020-21 during the global COVID-19 pandemic, which also altered the Indianapolis 500 in those same years. This meant that I saw very few other researchers during my drive to complete this research due to working in shifts, just as during the 2020 Indy 500 fans were not allowed to attend. Still, I had a core team that helped me to finish this race. Bob Landick, my advisor, acted as a type of engineer, planning with me the set-up of my experiments. Rodney Welch acted as a last-minute gracious sponsor and advisor, opening his lab to me to allow me to work with CFT073, even during COVID

restrictions. Kevin Schwartz acted as my driver's coach, providing me with protocols to genetically manipulate CFT073 to answer the experimental questions I wanted to ask, allowing me to finish the race. Michael Wolfe acted as an additional driver coach, with an emphasis on technical expertise: he provided a starting set-up for analyzing the data that allowed me to finish the race. Finally, Rachel Mooney was the one high-profile fan allowed into the race of this experiment; she was one of the few people I saw during the lonely hours of 4am-2pm when we were forced to work in shifts and provided great encouragement as I tried to finish the race.

Before I tell you the results of this race of the first chapter of my thesis, I need to tell you a bit more about the set-up and how I qualified for this experiment. Going into the race, we hypothesized that H-NS and StpA (one of those proteins that strengthens bridging in the lab, Figure 5.4) function similarly to how they function in lab strain *E. coli*: they silence gene expression and thus prevent RNAP accessibility. We also knew that a key elongation enhancer protein called RfaH was essential for the bacteria to infect humans.[10] We wanted to investigate how RfaH and H-NS may interplay to control transcription elongation, with the hypothesis that RfaH may overcome H-NS based silencing, allowing the bacteria to turn on certain virulence genes that could harm humans.

5.4.2 Race Strategy (Methods): Bacterial Genetics and ChIP-seq

The first technique I used to ask questions about how H-NS functions in CFT073 was genetic manipulation of the bacteria's genome to ask questions about how H-NS functions. This meant deleting the DNA sequence that encodes for the proteins of H-NS and StpA. I can compare the genome where I have deleted sequences to a reference, or control, of the normal (what we call wild-type) genome. This allows me to compare how RNAP moves on DNA in the presence and absence of H-NS and StpA. I also could delete the elongation enhancer, RfaH, in

combination with these other deletions so I could compare how transcription changed when different factors were present or deleted. This technique is like how race car engineers add or remove parts of the car to try to find more speed; comparing a run with a part to a run without a part helps the team to learn what the part does to the overall speed of the car.

I then used a method called ChIP-seq to monitor where proteins were bound on average on the DNA. This is like the scoring monitors they have along the racetrack which tell the fans where the race cars are positioned relative to another race car in the race. Overall, ChIP-seq tells me on average where RNAP, H-NS, RfaH and StpA are bound. I can also infer how many proteins are bound to the DNA by this method. Armed with these two main techniques, I had qualified for the Indy 500. Now I will tell you the results.

5.4.3 Race results: RfaH can help RNAP to overcome H-NS based elongation silencing

The results of this race were quite exciting; it was not a single-file race, but rather a race with many twists and turns, a few caution periods to re-analyze the data, one red flag to stop the race, and then a strong sprint finish to submit the paper.

What we found was that H-NS and StpA bound nearly 18% of the bacterial chromosome, largely on genes that encode for some sort of virulence function, like toxin secretion that can kill human cells and thus cause disease. RNAP at most of these virulence genes had very low occupancy, consistent with H-NS preventing RNAP from passing through (*i.e.* H-NS and StpA create a traffic jam on the DNA in the cell).

However, at a few operons throughout the genome that encoded for virulence genes, RNAP occupancy was quite high; RNAP could drive through the traffic jam! How could this be? What we found was that one of these operons was a known recruitment site for the elongation

enhancer RfaH, which binds to RNAP and helps RNAP to move. We had a new hypothesis: could RfaH help RNAP to drive through H-NS?

To test this, we used our ChIP-seq method to monitor where RfaH was bound to RNAP. RfaH was only localized to four places in the genome. All these loci had high RNAP levels, consistent with RfaH helping RNAP to transcribe through these loci. And very surprisingly, all these loci had H-NS and StpA bound! This means that when RfaH was bound to RNAP, RNAP could effectively transcribe through the H-NS filament!

To test this question, we deleted the DNA sequence encoding for the protein RfaH. When RfaH was deleted, sure enough, H-NS silencing took over. This means that RfaH helps RNAP to make it through the traffic jam of H-NS at key virulence loci. RfaH essentially acts as a police escort through this traffic jam.

5.4.4 Post-race perspective (model and significance)

The Indianapolis Motor Speedway is the pinnacle of innovation: stemming from the first rear-view mirror on a car in 1911. The Indianapolis 500 is also filled with thrill-seeking drivers who to many fans appear nuts. Up until 2012, drivers at the Indy 500 drove with an open cockpit (think a convertible without a windshield) (**Figure 5**). This allowed the drivers to increase their speed by improving aerodynamics and reducing drag. However, this also means that a crash with flying debris (parts of the car) could be detrimental to the progress of the racing car moving forward (**Figure 5**). If debris flies into the cockpit of a car, this will slow the car and then force retirement of the car from the race.

I liken debris slowing an open cockpit car to H-NS slowing RNAP. When RNAP is alone, without any proteins like RfaH binding to it, RNAP is slowed when it tries to transcribe

through H-NS. H-NS forces premature termination of RNAP, just as debris flying into an open cockpit forces premature termination of a race car from the race (**Figure 5**).

So, how have bacteria (like the cars of the Indianapolis 500) evolved to overcome this H-NS debris field? The race car engineers took a relatively simple approach, the addition of a windshield like attachment to the car called an aeroscreen (**Figure 5**). The aeroscreen protects the cockpit and the driver from flying debris, thus preventing premature retirement from the race. RfaH binds to RNAP to help RNAP to transcribe through the H-NS filament, just like an aero screen protects the race car driver, increasing the odds that RNAP finishes the race.

The aeroscreen has saved at least five drivers' races (and lives) since its implementation. By contrast, RfaH in CFT073 clearly aids RNAP to transcribe through key virulence genes, such as toxin secretion, which can harm humans. RfaH may aid the ability of bacteria to cause disease in humans by preventing H-NS disruption of RNAP progression, and thus may be an excellent protein to target with drugs to prevent disease in humans.

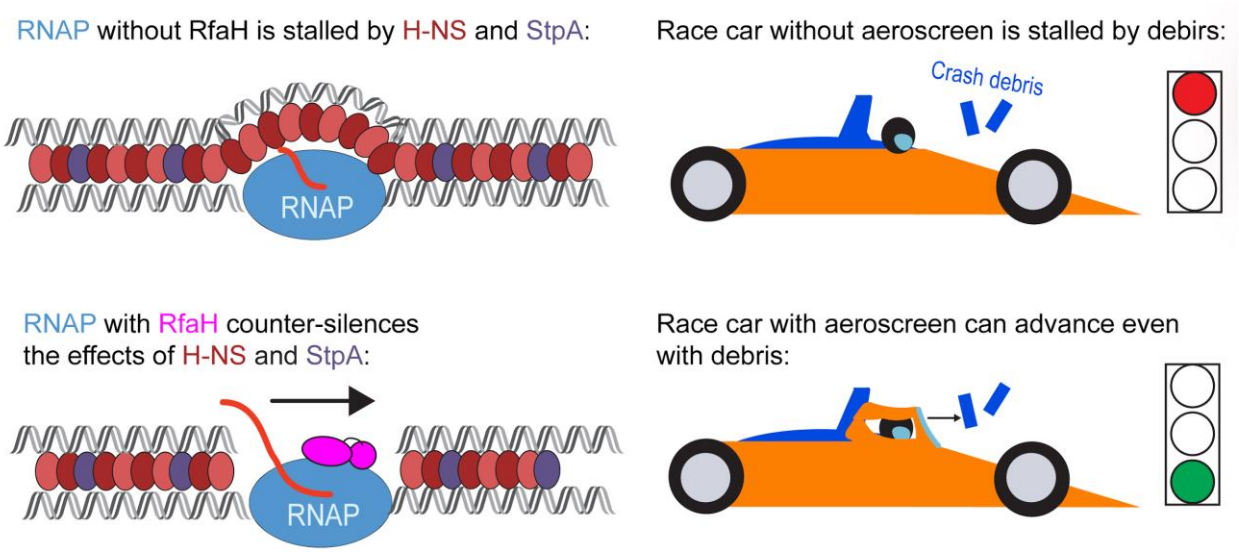

Figure 5.5 Model of RfaH effects on H-NS filaments.

Top Left: H-NS and StpA (red and purple circles) impede the progress of RNAP (blue) elongation on DNA in CFT073 pathogenic *E. coli*. Similarly, debris from a crash (like H-NS) can impede the progress of a race car in the Indy 500 without an aeroscreen protection. Bottom left: In the presence of RfaH (pink), RNAP can efficiently transcribe through H-NS and StpA filaments. Similarly, a race car with an aeroscreen (bottom right), which protects the driver from crash debris, is more likely to finish a race despite the presence of crash debris

5.5 What does H-NS look like?

5.5.1 How can we take a clear picture of a structure that is moving?

Next, we wanted to understand H-NS function and ability to disrupt transcription by getting a clear structure (or picture) of H-NS bound to DNA. What residues interact with each other? What residues interact with DNA? Can we find structural features that distinguish a bridged filament from a linear structure? Knowing the answers to these questions helps inform how H-NS works in the cell.

To do this we wanted to use a technique called Cryo-Electron Microscopy (see below). To use this technique, we had to do a team merger to gain a technical advantage. We merged labs with a collaborator CJ Lim and his graduate student Kaitlyn Abe. As a team, we attempted to solve the structure of an H-NS.

Our hypothesis was that an H-NS structure could be solved if we carefully manipulated the DNA template in the structure. We thought that creating an "ideal" DNA sequence with repetitive nucleotides that H-NS seems to like (or is frequently found bound to in the cell) may increase our chances of getting an interpretable picture of H-NS.

5.5.2 Race strategy (methods): Protein Purification and Cryo-EM

To complete this race, we needed two key techniques: protein and DNA purification and cryogenic electron microscopy. In the previous race, I told you about how we investigated H-NS and RfaH in cells, meaning all the proteins that are produced in *E. coli* could interact with the material used for experiments. To solve a structure, we need as minimal a system as possible. Thus, we purified H-NS and the DNA template from all other material present in the cell. This strategy sort of parallels the work that goes into racing car development. Often, engineers focus

on how a minimal part of the race car alters drag or speed in a wind tunnel to understand the bigger picture of the race car.

In order to look at H-NS bound to DNA, we used a technique called cryogenic-electron microscopy (Cryo-EM). In cryo-EM, we took our protein and DNA and then froze the complex at cold temperatures. These cold temperatures preserve the structure of the protein in multiple orientations. Then an electron can be shot through the protein to produce a reflective image of the protein. These images produce thousands of different 2-D structures which can then be averaged together to create a 3-D picture, or structure of a protein.

5.5.3 Race results: H-NS filaments are disordered even on an ideal DNA template

When I attend the Indy 500, I often like to take pictures of the cars as they flash in front of me at 240+ mph to preserve the memory. Usually, to my dismay, the images result in a blurry mess. I can rarely make out the details of the cars, the numbers, the sponsors, or even the drivers.

We knew going into this project that trying to solve a structure of an H-NS filament would likely result in a blurry mess of an image. Other studies that monitor where the parts of the protein were oriented on DNA illustrated that H-NS was highly dynamic (moving) when it binds to DNA.[11] So, we had to think of a way to engineer the components we put into the structure to make it move less, so we could get a clearer picture.

To do this, I manipulated a piece of DNA so that instead of having random orders of ACTGs, as found in the chromosome, I had evenly spaced sequences to try to force the structure of H-NS to bind to one part of the DNA. This would increase the likelihood of being able to take a picture of the protein, because the protein would not be moving around. Unfortunately, it seems that despite our “ideal sequence,” H-NS filaments are just still too disordered to solve a structure. We tested multiple filaments, which all seem to produce “blurry” structures. The notion that the

filament is still disordered even on a synthetic template may provide crucial insight into H-NS function.

5.5.4 Post-race perspective (model and significance)

Overall, we report that H-NS filaments are disordered on various DNA templates. This disorder prevented us from producing a high-resolution "picture" of H-NS filaments. All templates tested produce blurry structures, likely because H-NS forms disordered (or moving) structures on these DNAs. In the end, our goal was a picture of a static protein and DNA complex, but we are left with a blurry structure (Figure 5.6).

Although we plan to test other DNA templates in the future to see if we can get H-NS to be more static, it appears that H-NS filaments may function well when disordered and flexible in the cell. For example: racing cars can be static and still before the race, but it is in the power of movement at 240+ mph that racing cars produce their most exciting entertainment for fans. They function to move. H-NS flexibility may allow H-NS to better silence gene expression, for example, by being able to re-bind to DNA in a different conformation than previously.

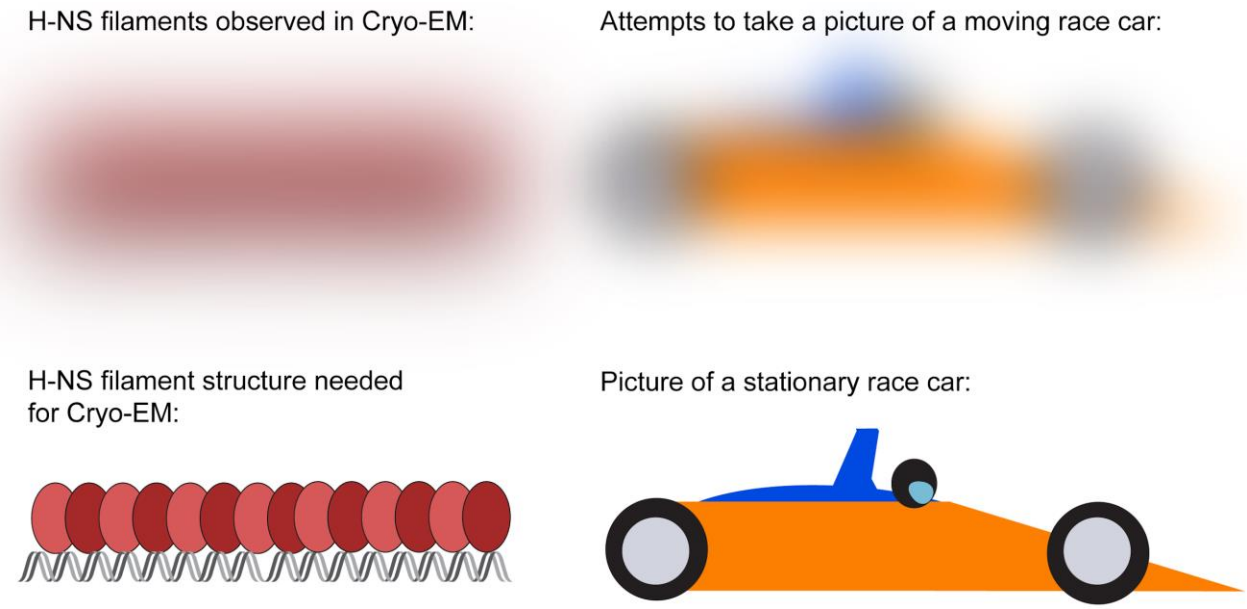

Figure 5.6. H-NS filaments as observed in Cryo-EM.

Top left: H-NS bound to DNA is quite flexible and disordered in Cryo-EM, such that when a veraging the images together, a blurry image is produced. This is similar to attempts to take a picture of a moving race car (top right). Bottom: goal is to produce the ideal conditions such that it produces a clear final picture, similarly to when taking a picture of a stationary race car.

5.6 Do other proteins strengthen the ability of H-NS to impede transcription elongation?

5.6.1 What is the role of StpA, Hha, and YdgT in H-NS based silencing of elongation in cells?

Next, we investigated how partners of H-NS might mediate silencing in the cell. I previously told you about our investigation of H-NS and StpA function in the cell in pathogenic bacteria. But has the cell evolved other proteins that strengthen H-NS based silencing? We are looking to find team members of H-NS that aid H-NS to accomplish its goal of silencing gene expression. Identifying and understanding how these team members work will help us to think about how we can make H-NS a more effective silencer of virulence gene expression, in turn preventing harm in humans.

We had knowledge of these players from previous studies done with purified DNA and proteins, as well as the first race of my thesis. The three additions to the H-NS team that we investigated are StpA, Hha, and YdgT. These proteins can interact with H-NS (*i.e.* attach to H-NS)[5, 6, 12, 13]. *In vitro,* these proteins seem to strengthen the ability of H-NS to silence gene expression.[5] They do so, we think, by forming bridged conformations of H-NS on the DNA.[5, 6] These bridged conformations are more likely to impede RNAP transcription and cause premature termination from the race of transcribing. We wanted to ask how these proteins functioned in the cell. We hypothesized that Hha, YdgT, and StpA function to increase bridging of H-NS in the cell. We sought to test this hypothesis with various methods in the cell, including some methods I previously described.

I needed to add team members of my own to investigate how members of the H-NS team aid H-NS to silence elongation of transcription in the cell. Thus, in this race, I was acting more like a stunt driver in an endurance race, such as the 24-hours of Le Mans. I took multiple shifts, genetically manipulating strains and performing assays to test where H-NS and RNAP were

distributed when YdgT, Hha, and StpA were present vs. when they were not. Two post-doctoral researchers, Dr. Mike Wolfe and Dr. Mike Engstrom, acted as additional drivers on the team; tackling other questions in this story, such as if RNAP slowed (paused) more frequently when these proteins were present vs. when they were not. Finally, we even had international drivers join our team in the form of Dr. Mounia Kortebi and Dr. Vicky Lioy, from the Institute for Integrative Biology of the Cell in Paris, France. Mounica and Vicky are pioneers of using a method called Hi-C for bacteria, which helps us to monitor where DNA contacts (in the form of bridging) occur in the cell. This helps us to understand if changes that we observe in the form of transcription are not only because H-NS is less apt to silence gene expression, but also because H-NS is less bridged in the absence of Hha, YdgT, and StpA.

To tackle a question this large, we combined multiple methods together to ask if Hha, YdgT, and StpA strengthen H-NS silencing of elongation by bolstering H-NS bridging. Our hypothesis was that, based on our *in vitro* results, we should see a decrease in bridging and effective silencing in the absence of Hha, YdgT, and StpA.

5.6.2 Race strategy (methods): ChIP-seq, NET-seq, and Hi-C

We used several unique combined strategies to tackle this question in cells. First, we used ChIP-seq, which, as aforementioned, tracks on average where proteins are distributed on DNA in the cell. With this method, we can learn where RNAP and H-NS are bound to DNA in the presence and absence of StpA, Hha, and YdgT. I used the analogy of a scoring tower or running race order earlier—the same analogy holds true in this question, as we essentially want to see where the proteins are located on the DNA so we can compare their locations in the presence and absence of the factors we are testing.

Unique to this experiment is the addition of a parallel Hi-C study with the same bacteria used for ChIP-seq. Hi-C allows us to map where DNA contacts each other, creating a 3-D map of where the DNA is orientated relative to a neighbor DNA. Combined with ChIP-seq, we can make conclusions about if a protein binding to DNA promotes close contact (or a bridge, bringing two DNAs together). If ChIP-seq is like a fan looking at the running order of drivers in the race on the scoring tower, Hi-C combined with ChIP-seq is like watching the race AND knowing the running order of the race. One gets a full "picture" of the contacts and distribution of proteins by combining these methods.

Lastly, we added a final method called NET-seq, which maps how much pausing of RNAP occurs where H-NS and its partners are bound. NET-seq essentially measures the amounts of stopping and starting of cars in a traffic jam. From this method, we get a direct view of not only if RNAP elongation is perturbed by H-NS and its partners, but also how much elongation is perturbed.

5.6.3 H-NS appears to more effectively silence RNAP elongation with the aid of StpA, Hha, and YdgT

The results of this race were surprising and still under review *(i.e.* we are still analyzing the data and deciding what additional experiments might be required). Overall, it appears from our ChIP-seq experiments that H-NS distribution does not change in the presence or absence of StpA, Hha, and YdgT. This means that H-NS can bind to the same DNA sequences regardless of StpA, Hha, and YdgT expression.

Interestingly, it seems that in the absence of StpA, Hha, and YdgT, RNAP can progress a bit further along DNA compared to bacterial strains with H-NS, StpA, Hha, and YdgT. Although we do see a modest increase in transcription elongation in the strain lacking these factors, overall transcription levels where H-NS is bound remain quite low compared to other places across the

genome. H-NS alone therefore is still able to keep RNAP transcription relatively low (*i.e.* still able to silence transcription). These data suggest that the RNAP in the bacteria that contains H-NS without StpA, Hha, and YdgT would finish the race of transcription slightly ahead of the RNAP in the bacteria with all four proteins. However, the race would be a close and exciting finish.

Why is there slightly more effective transcription in the bacteria without StpA, Hha, and YdgT compared to the strain with all three proteins? Our hypothesis was that these partner proteins strengthened bridging of H-NS, which *in vitro* means that the combination of this protein complex would further perturb elongation compared to H-NS alone. However, we do not know what type of complex (bridged or linear) forms in cells. Hi-C allows us to make this comparison. What we observed is that H-NS in the presence of StpA, Hha, and YdgT promoted close contacts between DNA in the cell, like what we would expect if H-NS bridges DNA (*i.e.* brings two pieces of DNA together). When we deleted StpA, Hha, and YdgT, we saw that those contacts not only remained, but slightly increased. Thus, H-NS in the absence of these factors can still somewhat perturb elongation, likely because H-NS is still slightly bridged in the cell. H-NS alone can create an extreme traffic jam even without the aid of StpA, Hha, and YdgT.

5.6.4 Post-race perspective (current model and significance):

Currently, our model demonstrates that Hha, YdgT, and StpA promote bridged H-NS filaments to slow down RNAP transcription. However, even without StpA, Hha, and YdgT, H-NS appears capable of slowing RNAP. Thus, H-NS can act as an individual to perturb elongation in the cell. We can think of the results as H-NS-StpA-Hha-YdgT creating a a larger traffic jam (*i.e.* larger protein structure) (**Figure 5.7**). This combination of proteins can effectively perturb elongation. However, H-NS alone can still perturb elongation, likely because it can form the same structure

on DNA that blocks RNAP elongation (**Figure 5.7**). A comparison to the Indianapolis 500 race can be made by thinking of the two strains (with or without the factors) as different race car accidents. When many cars crash, debris flies off multiple cars and can impede the progress of another race car trailing behind the large debris field. The effects of Hha, YdgT, and StpA can be thought of as simply more debris; when bound to H-NS, H-NS filaments can indeed impede the ability of RNAP to finish the race of transcription. By contrast, think of a small car crash at the Indy 500, where just one small piece of debris flies off the crashed car. This small piece of debris still is likely to perturb the race car trailing directly behind it. Even one piece of debris can ruin the ability of a race car to finish the Indy 500. Similarly, it appears H-NS alone is sufficient to impede RNAP elongation, although a mess of debris in the form of StpA-YdgT-Hha will slow RNAP a lot sooner and with more certainty than H-NS alone.

These data reveal *i)* H-NS in cells mostly forms these bridged (traffic jam) structures on DNA and *ii*) under the conditions tested, StpA, Hha, and YdgT have a small, but notable, effect on transcription elongation in the cells. Testing the effects of these proteins in other bacteria would be worthwhile and likely yield more dramatic results on changes in transcription. We performed our experiments in the lab-strain K-12 *E. coli* where keeping certain genes silenced might not be as crucial for bacterial survival. However, in pathogenic bacteria, for virulence genes to be expressed, the bacteria must overcome not just H-NS, but also StpA, YdgT, and Hha. These proteins may therefore play a more enhanced role in bolstering H-NS based silencing. Having back-up plans to silence transcription in the form of StpA, YdgT, and Hha may help ensure that bacterial behaviors are kept off. Bacteria likely use these proteins to keep bad behaviors off because to turn on the behavior requires energy and takes resources away from

other parts of the genome. As scientists, we can learn how H-NS and its team members fine-tune gene expression in cells to learn how we, as humans, can control bacterial genomes.

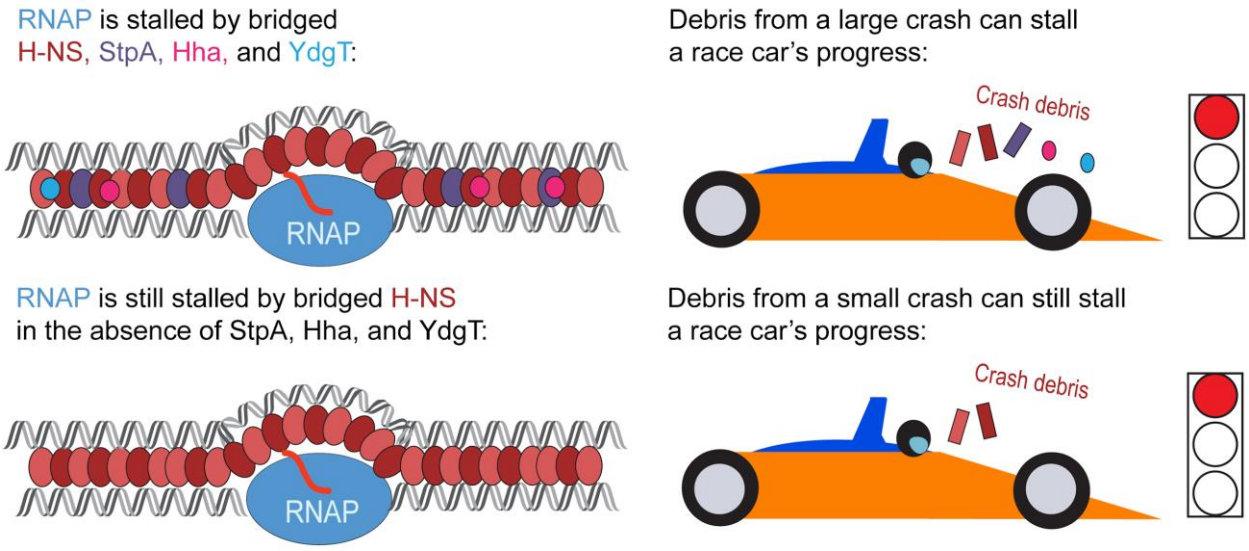

Figure 5.7. Model of effects of StpA, Hha, and YdgT on H-NS function.

Top left: Cartoon model of bridged H-NS (red and pink circles) filament with StpA (purple), Hha (blue), and YdgT (pink), which stalls RNAP from elongating in the cell, similarly to a race car encountering debris from a crash (top right). Bottom left: H-NS in the absence of StpA, Hha, and YdgT (red circles only) still can impede the progress of RNAP elongation by forming a bridged structure. Similarly, a race car encountering only a small amount of debris can still be stalled from finishing a race.

5.7 Post-race perspectives on my Ph.D.

In this chapter, I summarized three major scientific insights that I found during my Ph.D. I mastered many new methods in my quest to find answers to questions regarding how H-NS functions to silence transcription (*i.e.* turn off gene expression) in bacteria. Although many of these initial questions I sought to answer have indeed been answered, their answers bred even more questions about this protein and how it works in bacteria. My quest to find out, which only led to more quests to find out, led me to reflect on what I have learned from the Ph.D. about myself, and particularly to reflect on how my perspective on science has changed these past six years. To conclude, I discuss three major takeaways from my time as a Ph.D. student, including *i*) the importance of surrounding yourself with good people, *ii*) the importance of being comfortable with yourself and how you work, and *iii*) the importance of persistence and tenacity in science and in life.

5.7.1 No driver can compete in the Indianapolis 500 without a support team (or, the importance of those around me)

In the Indy 500, it takes a remarkable amount of people besides the driver to run a racecar in the race. The pit crew, engineers, strategists get the driver to and through race day. The team owner finds sponsorship (or funding) to pay for the people who service the car. Officials keep track of car speeds, inspect car parts, and investigate drivers if they are playing foul on the track. Public relations members employed by teams woo high-dollar sponsors on race day and shuffle drivers through throngs of eager fans with upheld sharpies waiting for autographs. The family and close friends of the driver wait anxiously, heads down, often near the pit box while their loved one risks their lives by driving 240 mph for 200 laps. All these people are essential when the driver succeeds, and even more essential in helping the driver persist if they fail.

In my Ph.D., I worked individually on most of my projects: I was the driver. But I would have been unable to finish the Ph.D. without many of the important people around me. My pit crew was my lab, my chief engineer was my advisor, my strategists were my committee members, and my technical officials were peer reviewers who reviewed my papers. All these people were crucial to prepare me for my experiments, help me through interpretation of experiments, and get me to the finish line of publishing papers. Where no analogy is needed is in the form of the importance of the support from my family and close friends. These people were by far the most important factors in my persistence in this Ph.D. program. And while I by no means was risking my life by driving a car around an oval track at 240 mph (I am not crazy), there were times during my Ph.D. where the support of my Dad and close friends were key to me continuing to pursue the Ph.D. I am forever grateful for the support from everyone who has crossed my path during this journey. I leave the Ph.D. with a renewed clarity on the importance of family and close friends, both of whom make my life so much richer.

5.7.2 Saving fuel can lead to a surprise Indy 500 win, but so can 4-wide crazy passes (or, learning to be comfortable with how I tackle work)

My favorite driver is Alexander Rossi. In 2016, as a rookie, he won the Indy 500 in shocking fashion by crossing the finish line with a nearly empty gas tank. He literally won a race known as "the pinnacle of speed" by going consistently slower than any other car. Early in the 2016 Indy 500, Rossi was disciplined enough to know that he could not win the race on pure out-right pace—the other cars were simply faster. So about mid-way through, he and his team devised a plan that would require Rossi easing up on the gas, purposefully going slower than the other cars, so he could save enough fuel to take one less pit-stop than the rest of the field. This meant that by the end, when all the other cars needed to pit for a re-fuel one last time (losing time in the pit),

Rossi could stay out on the track and literally coasted to the finish line (he ran out of fuel one turn later, was stalled on the track and had to be towed into Victory Circle).

Since 2016, Rossi has been extremely competitive at the Indy 500 using a different strategy: racing like a maniac. He is one of the most exciting drivers to watch on track because he makes what we racing fans call "dive-bomb" passes, placing his car where no other car can go on the track. To make these crazy passes, which may seem out-of-control, he must have *complete* control of the car to avoid crashing. He makes gutsy moves, gets mad when others critique him, and leaves every race with the cool confidence of someone who knows that other drivers cower when they see him in their rear-view mirrors. He is the epitome of a driver that can do both: master endurance and sprint. And he really does not care if you prefer one or the other, he is going to do what he wants.

Common advice that was given to me throughout my Ph.D. was that the Ph.D. is a marathon, not a sprint. That motto never really caught on for me, but I know it does to others. What I learned is that at the end of the day, I must be most comfortable with the work I put in. Most of my Ph.D., though a marathon in length, was an extreme sprint. I rarely saved fuel. I had a lot of tasks to juggle, I worked a lot, I sacrificed a lot, and I would not say I was "calm" throughout the race. Every day of my Ph.D. had value to me and required concentrated thought and time. And I think on finishing my Ph.D., I have a big sense of relief in saying that I am okay with how I tackled it. I am glad I worked so hard, even though I was quite frankly almost always burned out. And I think through the journey I can better recognize that people work in different ways: some people may want to save fuel, some may want to nearly crash, and that is part of the journey. In science, you work with people of such vastly different personalities, all of whom tackle questions in different ways. I leave my Ph.D. with more acceptance towards others'

different approaches—and, importantly for my own growth, more acceptance for my own approach. I realize that my relationship with work, like Rossi's driving style, can evolve from a sense of urgency to a more strategic, slower-paced effort. And although I plan to make saving fuel more of a priority in my future career, my Ph.D. has taught me that both approaches can win Indy 500s and get me to the finish line.

5.7.3 People DO remember which drivers see the checkered flag (or the importance of persistence in finishing the Ph.D.)

The Indy 500 is a long race, filled with ups and downs for many drivers. Sometimes, a mishap occurs early in the race that might seem like a catastrophe to the driver. A slow pit stop, a tire that takes too long to take off the car, fuel that will not flow: all of these bad-luck events are common and can take a driver from first place to dead last. What is great about the Indy 500 is that these drivers, if they keep trying, are often able to come back from what seemed like the end of their race by simply persisting. They can do this because the Indy 500 has a lot of crashes. Sometimes, these crashes are so damaging to the car that the driver must retire and does not finish (DNF), and this allows the driver who previously was in the back of the pack from early bad luck to advance by attrition. There have been times when a driver was dead last on lap 100 and in the top five cars by lap 200, all just because the driver persisted.

What I learned most in my Ph.D. was the importance of persistence even during what seems like catastrophe. There have been several unlucky moments in my Ph.D., which, especially in my early years, seemed like they would be detrimental to the progress of my project. Through the advice of those around me, including Bob and my Dad, I was able to learn how to turn these unlucky moments into something positive, such as a learning experience, a new scientific question, or a new way to grow as a person. I am not saying that these positive changes happened immediately. I, after all, do not have the willpower or heart rate of a race car

driver after a bad pit stop (and I already told you my favorite driver is the cantankerous Alexander Rossi!). But throughout my Ph.D., my perspective on how to look at the seemingly awful has shifted. I have learned various life lessons throughout graduate school, but by far the most important has been that keeping my head down and continuing to race is the most likely avenue to success. Finishing is more important than dwelling on the bad. Pessimism can always be replaced by hope (and likely another experiment).

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